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EXHIBIT 3

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Regulation of 5-Aminolevulinate Synthase mRNA in Different Rat Tissues

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cDNA clones for rat liver 5-aminolevulinate synthese have been isolated and used to examine mRNA levels in different rat tissues. Northern hybridization analysis of total RNA from various rat tissues showed the presence of a single 5-aminolevulinate synthese mRNA opecies of estimated length 2.3 hiloberes. Primer extension and RNace mapping studies indicated that the mRNA is identical in all tissues. Highest basal levels were com in liver and heart. Administration of hemin to rate reduced the basal level of this mRNA only in liver but the heme precureor, 5-aminolevulinate (or its methyl ester), repressed the basal levelo in liver, hidney, heart, testis, and brain. The drug 2-allyl-2-isopropylacetamide increased the mRNA level in liver and kidney only while human chorionic gonadotropin hormone elevated the level in testis. Administration of the home precursor 5-aminolevulinate prevented these inductions. Nuclear transcriptional run-off experiments in liver cell nuclei showed that 2-allyl-2-icopropylacetamide and 5-aminolevulinate exert their effect by altering the rate of transcription of the 5-aminolevulinate synthese gene. The results indicate that a single 5-aminolevulinate synthase mRNA is expressed in all tissues and that its transcription is negatively regulated by heme.

5-Aminolevulinate synthase catalyzes the first step of the heme biosynthetic pathway and in the liver at least is ratelimiting (reviewed in Refs. 1 and 2). The enzyme performs a househeeping function since all animal cells synthesize their own heme for mitochondrial cytochromes and other cellular hemoproteins. The enzyme level is normally very low in animal tissues but is greatly elevated in the liver of experimental animals following administration of a wide variety of porphyrinogenic drugs such as AIA' and phenobarbital (3). This biochemical response mimics the acute porphyria diseases in man where hepatic 5-aminolevulinate synthase levels are elevated during clinical attacks. Drugs which precipitate such attachs induce 6-aminolevulinate synthase levels in experimental animals. These same drugs also induce the synthesis of hepatic cytochrome P-450 proteins, which are involved in the conversion of foreign compounds to watersoluble derivatives.

Granick (3) first demonstrated that the end product heme prevented the drug-induced increase in hepatic 5-aminolevulinate synthase enzyme levels. Work in our laboratory and elsewhere (reviewed in Ref. 1) has suggested that heme acts by repressing the synthesis of 5-aminolevulinate synthese mRNA, but there has been no direct proof of this. Current evidence favors the hypothesis that the porphyrinogenic drugs act by inducing synthesis of cytochrome P-450 apoprotein which results in a reduction in the heme concentration, thus indirectly leading to an increase in 5-aminolevulinate synthase mRNA levels (1). Erythroid 5-aminolevulinate synthase is not induced by porphyrinogenic drugs (2), and this finding has led to the proposal that erythroid and hapatic 5-aminolevulinate synthases are distinct enzymes (4, 5). Indeed, it has been proposed that a multigene family exists for 5-aminolevulinate synthese with different mRNA species synthesized in different tissues (6). However, recent work suggests that 5-aminolevulinate synthase mRNA is the came in the liver and erythroid spleen of mice (7).

We are interested in determining at the molecular level how heme regulates the gene for 5-aminolsvulinate synthase in liver and other tissues. cDNA (6, 9) and genomic clones (10) for chicken 5-aminolevulinate synthase have been isolated in this laboratory. In this communication we report the isolation of cDNA clones for rat liver 5-aminolevulinate synthese and provide strong evidence that the mRNA is identical in all rat tissues examined. We have established that drugs increase the level of 5-aminolevulinste synthese mRNA in a tissue-specific fashion and that heme represses levels of this mRNA in all tissues examined except erythroid spleen. Unequivocal evidence has been obtained that the altered levels of hepatic 5-aminolevulinate synthase mRNA observed after drug or heme administration are due to changes in the rate of transcription of the gene.

EXPERIMENTAL PROCEDURES

Materials-AIA was a generous gift from Roche, Australia. Hemin (ferriprotoporphyrin IX chlorids) was supplied by Porphyrin Pred-(ferriprotoporphyrin IX chlorids) was supplied by Porphyrin Products, Logan, UT. A chicken \$\text{\text{\$-}}\actin cDNA clone in pBR322 (insert 1.8 kb) was provided by \$S\$. Dalton and a chicken serum albumin cDNA clone in pBR322 (insert 2 kb) by \$A\$. \$H\$. Hobba. All other materials were purchased from sources proviously described (9, 11).

Treatment of Animals—Male albino Winter rate (200 g body weight) were given injections of AIA (80 mg) subcutaneously, 5-

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¹ The abbreviations used are: AIA, 2-allyl-2-isopropylacetamide; SDS, codium dodecyl culfate; HCG, human chorionic gonadotropin hormone; RNase, ribonuclease; hb, hilobase(s); bp, base pair(s).

aminolevulinate or ita methyl ester (333 mg) via the intraperitoneal cavity, or hemin (1.5 mg) via the tail vein. For induction with AIA over 12 h, a escond injection of AIA was given at 7 h. 5-Aminolevulinate or hemin was given immediately after AIA injection. Rats were treated with HCG over 48 h by repeated subcutanoous injections (20 unito) at 12, 24, and 36 h, 5-aminolevulinate was administered at 36 h and total mRNA isolated after a further 12 h. To obtain anamic rate, phenylhydrazine HCI (1.5 mg) was injected subcutanoously on 5 consecutive days and mRNA isolated on the sixth day. Anamic rats were treated with 5-aminolevulinate 12 h prior to death.

were treated with 5-minolevulinate 12 h prior to death.

For developmental studies, Sprague-Dawley rats were used. Fetal eggs were estimated from the time of mating, males were placed with females overnight and the following morning taken us day 0 of gestation. Total RNA was isolated essentially by the method of Liu at al. (12) from model individuals of at least 2 litters for each age.

gestation. Total RNA was noisited easierably by the intention of al. (12) from peopled individuals of at least 2 litters for each age.

Construction and Screening of a Rat Liner cDNA Library—Liver poly(A)* RNA from ruts treated with AIA was used to construct a cDNA library. Double-stranded cDNA was synthasized by the procedure of Gubler and Hoffman (13), and DNA greater than 1600 by was annealed with dG-tailed Pull-digested pBR322 and transformed into Eccherichia call MC1051. Recombinant planmids were coround with a mixture of "P nich-translated probes comprising the four Pull inserts from the chicken 5-aminolevulinate synthase clone, p105B1 (9).

The clone p101B1 was requested by disjection of plasmid with either Hpall, Pall, SusSal, or Teql and fragments sequenced by the method of Sungar et al. [14].

method of Sangar et el. (14).

Analysis of RNA—Total RNA was isolated from int tissues by the guanidine hydrochloride entraction procedure of Brosker et el. (15).

Poly(A)* mRNA was prepared by oligo(dT)-cellulose chromatogra-

phy.

For Northern blot analysis, RNA was electrophoresed in 1.0% agarose gale containing 1.1 M formaldehyde as described (11). RNA was transferred to nitrocelluloco fitters (BA85 from Schleicher & Schuell) and hybridized to the "P nich-translated rat 5-aminolevulinate synthese cDNA close p101B1 (10 ng/ml) in a solution containing 0% formamide, 5 × SSPE (0.9 M NsCl, 50 mM codium phosphate buffer, pH 7.0, 5 mM EDTA), 5 × Denhardt's (0.04% Ficell, 0.04% polyvinylpyrrolidone, 0.04% bovine earum albumin), 0.1% SDS, 0.6% codium pyrrophosphate, and 200 µg/ml denatured salmon aparm DNA at 42 °C for 20 h. Filters were washed finally in 0.1 × SSPE containing 11% SDS at 60 °C for 40 min. For low stringency conditions filters were hybridized as above and washed in a colution containing 2 × SSPE and 0.1% SDS at 50 °C for 40 min. Molecular size markers consisted of DNA frarments generated by Acri digestion of pBR322.

SSPE and 0.1% SDS at 60 °C for 40 min. Molecular size markers consisted of DNA fragments generated by Acri digestion of pBR322.

RNA was denatured and bound to nitrocellulose filters using either a clot or det blot apparatus (Schleicher & Schuell). Hybridization conditions were as described above. The amounts of RNA in Northern and alst blots were quantitated using an LKB laser denaitometer. Primer extension analysis using poly(A)* RNA from different rat

Primer extension analysis using poly(A)* RNA from different rat tissues and 5'-3'P-labeled synthetic primers complementary to the coding strand of p101B1 was carried out according to the method of McKnight at al. (16). The extended products were analyzed by electrophoresis on 8 M ursa, 6% polyscrylamide gels with a dideoxy sequence ladder of M13 bacteriophage DNA as also standards or ³⁰P-labeled Hpall fragments from pBR322.

RNace Mapping—The three Pal restriction fragments of p101B1

RNase Mapping—The three Pat1 restriction fragments of p101B1 (css Fig. 5) were individually subrloned into Pat1 cut pGEM-1 vector (Promega Biotech). Two probso, A and E. contained the 5' and 3' Pat1 fragments of p101B1, respectively (css Fig. 5). The plasmid containing the largest Pat1 fragment of p101B1 was further digested with appropriate restriction ensymes to generate three subclones of suitable size for RNase mapping, restriction ensyme removal of a BamHI fragment (one BamHI site in polylinher) generated a clone for the protection of a 364-bp BamHI-Pat1 fragment (probs D). Digestion of the plasmid with BgIII and HindIII (polylinher site) allowed the religation of a clone for the protection of a 631-bp Pat1-BgIII fragment (probs B). Probe C was generated by directionally cloning the 639-bp BamHI/SaII fragment in a BamHI/SaII cut pGEM1 vector.

RNA probes uniformly labeled with [*P]UTP were generated in vitro from the five recombinant pGEM plasmids using either T7 or SP6 polymerase as described (11). Specific activities of about 10° cpm/µg RNA were routinely obtained. Full-length transcripts were isolated on a 5% polyacrylamide equencing gel and eluted in 500 mM ammonium creatate, 1 mM EDTA, 0.5% SDS for 3-6 b at 37 °C. RNase mapping using RNase A and T1 was carried out as described previously (11) and protected fragments analyzed following electro-

phoresis on a 5% polyacrylamide requencing gel and autorodiography. Nuclear Transcription Assays—Nuclei were isolated from rat livar as described by Schibler et al. (17). The transcription reactions contained 100 mm Tris-HCl, pH 7.9, 80 mm NsCl, 5 mm MgCls, 1.5 mm MnCls, 0.4 mm EDTA, 0.1 mm phenylmethylaulfonyl fluoridz, 1.2 mm dithiothreitol, 30% glycerol, 2 μ m UTP, 1 mm each of ATP, CTP, and 6 GTP, 100 μ Cl of $[a^{-c}P]$ UTP, and 1.5 \times 10° nuclei in a final volume of 150 μ l. These were incubated at 26 °C for 15 min, and "P-labeled RNA was extracted as described by Vannics et al. (18). Rats were induced with AIA for 4 h. When 5-aminolevulinate was administered, it was injected 10 h prior to AIA treatment and the nuclei prepared 4 h later. For quantitation of specific transcripta, 5 μ g of the appropriate cloned DNAs (double-otranded DNA from the chicken serum allumin and β -actin cDNA clones or single-stranded DNA from an M13 phage clone containing the 1.7-th Pu1 fragment of p101B1) were denatured and applied to a nitrocellulace filter using a slot blot apparatus. Filters were prehybridized in 1 ml of 50% formamide, 5 \times SSC, 10 mm Tris-HCl, pH 7.5, 1 mm EDTA, 0.13 seedium pyrophexphate, 0.1% SDS, 100 μ g/ml K coil tRNA, and 0.2% seech of Ficoll, polyvinylpyrrolidone, and bovine cerum albumin at 52 °C coversight. Hybridization was carried out in the same solution with 2 \times 10° cpm of "P-labeled RNA for 72 h at 52 °C. Filters were vashed twice at room temperature for 30 min in 2 \times SSC, 0.1% SDS, 0.1% sodium pyrophosphate and then twice in 0.5 \times SSC, 0.1% SDS, 0.1% codium pyrophosphate as 65 °C for 60 min. The hybridization algnals were quantitated by laser denaitometric ceanning.

RESULTS

Isolation of Rat Liver 5-Aminolevulinate Synthase cDNA Clones—A cDNA library was constructed using poly(A)* RNA from livers of rate induced with the porphyrinogenic drug AIA. Size-selected double-stranded cDNA was used to construct a library of 4800 recombinant clones which were screened using a mixture of **P-labeled cDNA probes prepared from the four Pst I fragments of a previously isolated chicken liver 5-aminolevulinate synthase cDNA clone (9). Four clones gave positive hybridization signals, and the largest of these, p101B1, was sequenced. The esquence contained an open reading frame of 1929 nucleotides from nucleotides 17 to 1945 giving a predicted protein of 642 amino acids (Fig. 1). The sequence of the first 15 N-terminal amino acids of mature 5aminolevulinate synthase purified from the mitochondria of drug-induced rat liver (19) was determined and shown to be identical to that deduced from the nucleotide sequence of p101B1 from position 185 to 229. This shows that the glutamine (at nucleotide 185) is the N-terminal amino acid of the mature protein. Upstream from this codon there are three inframe d(ATG) codons. The d(ATG) codon at nucleotide 17 is assumed to be the initiation codon since it would result in the translation of a 5-aminolevulinate synthase precursor with a presequence of size 6 hDs in agreement with the size estimated from previous studies (19).

The deduced protein sequence of rat liver 5-aminolevulinate synthase precursor was compared with that of chicken (9) and mouse (7). The sequence of rat precursor was very similar to that of chicken, both in the N-terminal presequence (56 amino acids) and over most of the mature protein sequence. Surprisingly, the rat protein sequence showed less overall homology to the mouse enzyme, and indeed no homology existed within the presequence segments.

Northern Analysis of 5-Aminolevulinate Synthase mRNA— The size of 5-aminolevulinate synthase mRNA in different rat tissues was determined by Northern blot analysis. Total RNA was isolated from the liver, kidney, brain, and testis of untrested rats. Tissues were also enamined following treatments which are known to elevate 5-aminolevulinate synthase activity levels; for this total RNA was isolated from AIAtreated rat liver and kidney, HCG-treated rat testis, and spleen of rate rendered anemic by phenylhydrazine treatment.

The RNA samples were fractionated on a formaldehyde

Pto. 1. Nucleotide and predicted amino acid sequence of rat liver 5-aminolevulinate synthase. The cloud has 5'- and 3'-noncoding regions of 16 and 112 nucleotides, respectively. A possible polyadenylation signal ATTAAA is underlined, and the termination codon is attribad. The arrow indicates the cleavage site of the presequence.

| | 20 | 30 | 40 | 50 | 60 | 70 | B0 | 90 | 100 | 110 | 120 |
|--|--|---|--|---|--|--|---|--|--|--|--|
| COCACACTTTOCAG | N E 1 | V V E | | LE | T P Q | AFL | Q E A G | K 8 L | E F T | A Q H C | 7.4 |
| 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 |
| CATCATCCAACTEC | MM 1444 | WITH THE | ACTORION M | THENTHE | WWW.COCK | CACCITCAAAC | WCCCCTCC | METALTEN | micin | KTOOLANGO: | OC 4627 |
| # W E V | 5 A I P | | 1 4 5 1 | 5 A A | | | | | | | |
| CLACCAZZETTEETIC | 260 | 270 | 280 | 230 | 300 | 310 | 320 | 330 | 345 TTTG40CMT | 350 | 360 |
| QQAP | DESQ | M A Q | TPDG | TOL | PPG | H F .8 | P 8 T 8 | 0 5 5 | 6 5 K | CPFL | 4.4 |
| 370 | 380 | 390 | 400 | 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 |
| ACKSET MOCHEMO | OOC OC AC | ETCTTOOOL | MODELACTOR | CACCTICAC | MONETON TO | CASCAMITOC | ATOCTCICAG | CACAGAGOT | TOTTOMO | ENCTORTECT OF | ACCTY |
| | | | | | | | | | 580 | | |
| OUTCANTOCHAM | SOO COCATOGAÇA | S10 | 520 XXACTOCTGAA | 530 GAACTROEAS | 540 XACATCATG | SSO ACUMOTUMA | 960 220CAGAAAG | 570 LGIGICICA | ICTICTICAD | 590 ATAACTROLLL | 600 TOMACT |
| 7 H A E | | G P S | P L L E | WFQ | D 1 M | D K Q 1 | . , | * 5 B | 1 . 0 | D # L P | |
| 610 | 620 | 630 | 640 | 650 | 660 | 670 | 680 | 690 | 700 | 710 | 720 |
| COMPOSITION | D Y D W | FFE | K K I D | EKK | W D II | TTP | 7 7 2 7 | A H E | *** | FFR | 7.5 |
| 730 | 740 | 750 | 760 | 770 | 280 | 790 | 800 | 610 | \$20 | 830 | 840 |
| TEACTICACIDACE | EDETEATOR | CANTANTCAC | CIGIOCICIO | CTCGACTAN | CACTATUTA | DOCATGACTO | | MAINIGINA | DODGETONIA | ACACTICITANA | CACCA |
| | | _ | | | | | | | | | |
| 700100000TUCAG | 960 COGGAACTAG | B70 AMTATTICI | 680 CGAACGACGAA | 890 GTTCKATGI | 900 XAACTIXAG | 910 CACCACCTUC | 920 TGACCTICA | 930 TELEARCEAC | 940 | 950 Higherenties | 960 RIZETT |
| GAGA | GGTB | N 1 8 | GTSK | 7 # 4 | | QEL | PLH | CKD | AAL | L F S S | CF |
| 970 | 980 | 990 | 1000 | 1010 | 1020 | 1030 | 1040 | 1050 | 1060 | 1070 | 1080 |
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| 1090 | 1100 | | 1120 | 1130 | 1140 | 1150 | 1160 | 1170 | 1180 | 1190 | 1200 |
| 1000 | 1100 | 1110 | 1120 | | | | | | | | |
| GINING TOURS | ACASTESTOR | CANCELTER | AGAGAACTUTT | CACACATO | EACOCOTTOE | CTCOUCAGA | ROCTACCATTO | EARCHUR | DONTICATO | ATOCACCACTO | 10000 |
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agarose-denaturing gel and probed with ²²P-labeled p101B1. In all tissues, only a single 5-aminolevulinate synthase mRNA species was detected, estimated to be 2.3 kb in length (Fig. 2). In some tracks, very faint extra bands were visible and were probably due to ribosomal RNA. The results therefore show that there is no difference in size between 5-aminolevulinate synthase mRNA from either untreated or induced rat tissues.

It was also of interest to determine the 5-aminolevulinate synthase mRNA during fetal development. A single 5-aminolevulinate synthase mRNA species (2.3 kb in length) was detected at day 14 of gestation when the liver is largely erythroid in nature (20) through to the adult stage (Fig. 3). The level of mRNA peaked prior to birth at gestation days 20 and 21. This level then declined significantly at birth and in the adult had returned to the level seen prior to birth.

Primer Extension and RNase Mapping Studies of 5-Aminolevulinate Synthase mRNA from Rat Tissues—Further studies were carried out to determine whether 5-aminolevulinate synthase mRNA was identical in different tissues of rat. Of particular interest was the relationship of the nonerythroid to erythroid mRNA in view of the proposal that these mRNAs are distinct in the chicken (6). A $5'-\gamma^{-23}$ P-labeled 23-nucleotide oligomer complementary to the coding sequence of p101B1 from nucleotides 17 to 40 (Fig. 1) was used in a primer extension reaction on poly(A)* RNA isolated from tissues of untreated and treated rats (see legend to Fig. 4). With all

mRNA samples, two bands, 144 and 147 nucleotides in length, were seen following electrophoresis of the extension products and detection by autoradiography (Fig. 4). Such doublets have been observed previously and may be due to an artifact of the reverse transcriptsse reaction (21) or to initiation of mRNA synthesis at an alternative nucleotide. A second set of primer extension reactions was performed using a primer complementary to a sequence in the 5'-untranslated region of p101B1 from nucleotides 3 to 26. The rationale for this was that different mRNAs in different tissues may be homologous in their coding sequences but different in the 5'-untranslated region. With all tissues a doublet was observed with hands 108 and 111 nucleotides in length (result not shown). The results suggest that the 5'-untranslated region of the 5-aminolevulinate synthase mRNA from different tissues is the

As indicated earlier, the 5'-untranslated region of p101B1 is 16 nucleotides in length. The primer extension analysis indicates that in the mRNA, this region is 100 or 103 nucleotides in length, and p101B1 is, therefore, 84 or 87 nucleotides short of being full-length.

For RNase mapping experiments, five [a-2*P]UTP-labeled RNA probes complementary to rat hepatic 5-aminolevulinate synthase mRNA were synthesized by in vitro transcription of pGEM-1 plasmids containing appropriate restriction fragments of p101B1 (see Fig. 5). The probe sequences (A-E)

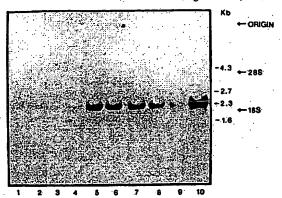


Fig. 2. Northern blot analysis of 5-aminolevulinate synthese mRNA in rat-tissues. Total RNA from tissues of untreated rate was electrophoresed on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. Molecular size markers consisted of DNA fragments generated by Acri digestion of pBR322. The positions of rRNA markers are shown. Lane 1, crythroid spleen (50 μg); lane 2, brain (50 μg); lane 3, testis (20 μg); lane 4, testis (HCG-treated rat) (10 μg); lane 5, heart (20 μg); lane 6, heart (AIA-treated rat) (20 μg); lane 8, lidinsy (50 μg); lane 9, liver (5 μg); lane 10, liver (AIA-treated rat) (50 μg); lane 9, liver (5 μg); lane 10, liver (AIA-treated rat) (50 μg); lane 9, liver (5 μg); lane 10, liver (AIA-treated rat) (50 μg); lane 9, liver (5 μg); lane 10, liver (AIA-treated rat) (5 μg).

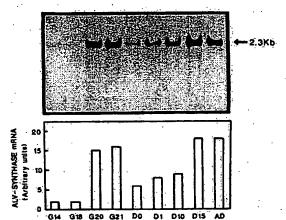
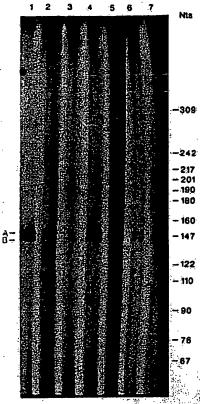


Fig. 3. Developmental profile of 5-aminolevulinate (ΔLV) synthase mRNA. Total RNA: {10 μg} from ret liver at various stages of development was resolved on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. The size of the mRNA was estimated as in Fig. 2; and mRNA levels were quantitated from the Northern blot by densitometric scanning and shown as bar graphs. Loadings of RNA were shown to be uniform by ethidium bromide staining of the gel. G, gestation day, D, day following birth; AD, adult, 12 weeks:

complementary to the mRNA were 73, 631, 699, 364, and 210 nucleotides in length, respectively, and spanned the entire mRNA except for 83 nucleotides not present in the cDNA clone at the extreme 5'-end and 90 nucleotides between the Bgill and Soll sites (see Fig. 5). Poly(A)* RNA samples were hybridized to these probes, and nonhybridized RNA was digested with RNase A and T1. The protected radiolabeled fragments were resolved on a 5% polyacrylamide sequencing



PIG. 4. Primer extension analysis of 5-aminolevullinates synthase mRNA in various rat tissues. A chemically synthesized 23-nucleotide oligomer was 5'-phosphorylated with (7-PplATP and used to primer extend on poly(A)" RNA from untreated rat liver (10 µg) (lane 2); hrain (50 µg) (lane 3); heart (10 µg) (lane 4); erythroid spleen (50 µg) (lane 7); Alt-treated rat liver (5 µg) (lane 5); HCG-treated rat liver (5 µg) (lane 5); Products were analyzed on an 8 m ures, 6% polyacrylamide gel with "P-labeled Hpoll fragments from pBR322 as size standards. Banis A and B size 147 and 144 nucleotides in length; respectively. No; nucleotides.

For liver and erythroid spleen mRNA, all five RNA probes were employed. Fragments representing full-length protection products for each probe were observed, establishing that these mRNAs are very likely identical. Mapping with probe E which spans the 3'-noncoding end of the liver mRNA revealed two bands smaller than the expected full-length product of 210 nucleotides (Fig. 5). These additional bands may be caused by heterogeneity in the lengths of the poly(A) talks of isolated liver mRNA. In other experiments only(A) talks of isolated with mRNA from liver, kidney, brain, heart and testis of untreated rists, from kidney of AIA treated rists, and from testis of HCG-treated rats. This probe fully protected the expected 631-nucleotide fragment in all the mRNA samples axamined (results not shown). In summary, this study, together with Northern blot and primer extension analyses, provides compelling evidence that 5 aminolevulinate synthase mRNA is identical in all rat tissues.

Measurement of Basal Levels of 5-Aminolevulinate Synthase mRNA in Rat Tissues—Total RNA was isolated from tissues of untreated rats, and 5-aminolevulinate synthase mRNA

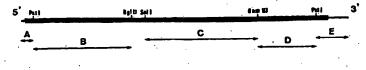
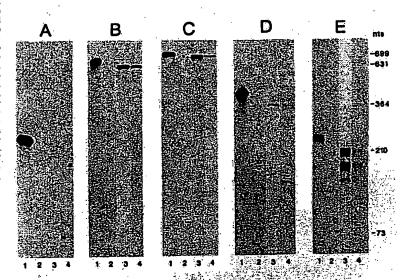
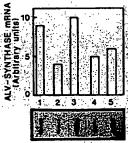


Fig. 5. RNase mapping of rat liver and erythroid spleen 5-aminolevulinate synthase mRNA. The cDNA clone, p101B1, is represented in the upper part of the figure with the coding and noncoding regions depicted as heavy and light lines, respectively. The double-headed arrows below show the relationship of the RNA probes A=E to p101B1. *P-Labeled RNA probes A=E to p101B1. *P-Labeled RNA probes WNA from AIA-induced liver (lane 3) or crythroid spleen (lane 4) and incubated with RNase A and T1. Probes incubated in the absence of poly(A)* RNA were either untreated (lane 1) or treated (lane 2) with RNase A and T1. Protected fragments were analyzed by electrophoresis and autoradiography as described under *Experimental Procedures.* The numbers displayed to the right of panel E correspond to the expected nucleotide lengths of the protected fragments. nis, nucleotides.

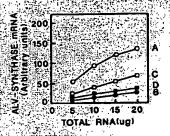




Pic. 6. Measurement of basal 5-aminolevulinate (ALV) synthase mRNA levels in rat tissues. Total RNA (10 μg) from tissues of untreated rats was applied as slots on nitrocellulose and hybridized to the nick-translated Pst I inserts of p101B1. 5-Aminolevulinate synthase mRNA levels were quantitated by densitometric scanning and shown as bar graphs. Lane 1, liver; lane 2, kidney; lane 3, heart; lane 4, brain; lane 5, testis.

amounts were quantitated. As can be seen in Fig. 6, the heart has the highest level of 5-aminolevulinate synthase mRNA, slightly above that of the liver, while in kidney, brain, and testis there is approximately half this level.

Effect of Hemin and 5-Aminolevulinate on 5-Aminolevulinate Synthase mRNA Levels in Untreated and Drug-treated Rats—The effect of hemin on liver 5-aminolevulinate synthase mRNA levels was first examined. Fig. 7 shows that



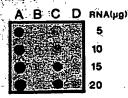


Fig. 7. Effect of bemin on 5-aminolevulinate (ALV) synthase mRNA levels in rat liver. Total RNA was isolated from rat liver 2 h after administration of AlA, hemin, or both. Amounts of total RNA (5-20 µg) were applied as dots on nitrocellulose and hybridized to the nick-translated Ppti inserts of p101B1. mRNA levels were quantitated by densitometric scanning. Lane A, AlA treated; lane B, AlA and hemin treated; lane C, untreated; lane D, hemin treated.

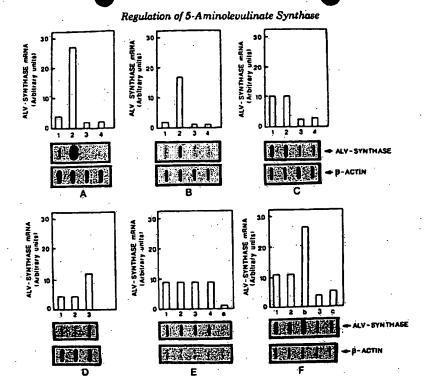


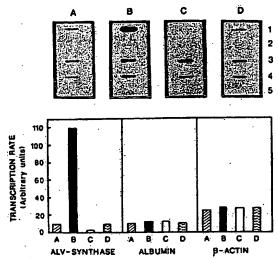
Fig. 8. Measurement of 5-aminolevulinate (ALV) synthase mRNA levels in rat tissues after 5-aminolevulinate treatment. Total RNA (10 μg) from tissues 12 h after administration of AlA or 5-aminolevulinate and from testis after HCG administration was applied as alots on nitrocellulates and hybridized to the nick-translated PstI inserts of p101B1. mRNA levels were quantitated by densitometric scanning and shown as bar graphs. Panel A, liver, panel B, kidney, panel C, heart; panel D, crythroid spicen; panel E, brain; panel F, testis, Lane I, untreated; lane 2, AlA treated; lane 3, 5-aminolevulinate treated; lane 4, AlA and 5-aminolevulinate treated. lane a, 5-aminolevulinate methyl ester treated; lane b, HCG treated; lane c, HCG and 5-aminolevulinate treated.

treatment of rats with the drug AIA over a 2-h period increased the level of 5-aminolevulinate synthase mRNA (Fig. 7, A and C). Administration of hemin either to drug-treated or untreated rats reduced the level of 5-aminolevulinate synthase mRNA to below that of basal levels (Fig. 7, B and D).

In similar studies it was found that hemin had no effect on the mRNA level in the extrahepatic tissues investigated (data not shown). It was possible, however, that injected hemin was not reaching or not entering the cells of these tissues. Anderson et al. (22) has reported that administered 5-aminolevulinate is taken up by many tissues and converted rapidly to heme. The effect of 5-aminolevulinate on 5-aminolevulinate synthase mRNA levels in different rat tissues was, therefore, studied in both normal and drug-treated animals. Of the tissues studied only liver and kidney showed induction by AIA; 5-aminolevulinate administration completely prevented the increase in mRNA levels in both tissues (Fig. 8, panels A and B). Similarly, basal levels of 6-aminolevulinate synthese mRNA in the liver, kidney, heart, and testis were reduced by 5-aminolevulinate to low levels (Fig. 8). In the case of brain, 5-aminolevulinate had no effect, but the methyl ester derivative of 5-aminolevulinate significantly reduced the mRNA level (Fig. 8, panel E) presumably because this compound can readily cross the blood-brain barrier. Erythroid spleen was an exception with 5-aminolevulinate treatment resulting in an increase in 5-aminolevulinate synthase mRNA levels (Fig. 8, In other experiments, it was shown that 5-aminolevulinate prevented the HCG induction of 5-aminolevulinate synthese mRNA in the testis (Fig. 8, panel F).

In all the experiments described here the level of β -actin mRNA in the tissues was quantitated (see Fig. 8, but not shown graphically) and was found to be essentially unchanged indicating that the response of 5-aminolevulinate synthase mRNA levels to AIA, HCG, or 5-aminolevulinate did not reflect a general cellular event. We also measured 5-aminolevulinate synthase activity in homogenates of all tissues examined above, and the amounts detected correlated closely with the changes observed in 5-aminolevulinate synthase mRNA levels (results not shown).

Hemin Acts at the Transcriptional Level in the Liver—The experiments described above show that in different tissues AIA and heme regulate the levels of 5-aminolevulinate synthese mRNA. We investigated whether, in liver, this reflected transcriptional control. Rat livers were removed, nuclei isolated, and gene transcription activities quantitated. In this in vitro system, it was established that incorporation by the nuclei of [a-xp]UTP into total RNA was linear for at least 30 min, and the extent of incorporation was similar in nuclei from untreated rats and rats treated with AIA, 5-aminolevulinate, or both. Additionally, a-amanitin (2 µg/ml) in the reaction mixture inhibited total RNA synthesis by about 40% and completely inhibited the synthesis of specific transcripts.



Pig. 9. Effect of 5-aminolevulinate on 5-aminolevulinate Pio. 9. Effect of 5-aminolevulinate on 5-aminolevulinate (ALV) synthase gene transcription. Rata ware treated with AIA for 4 b. 5-Aminolevulinate was administered 10 b prior to AIA, and I*PRNA subsequently was isolated from rat liver nuclei and hybridized to nitrocellulese filter-bound cloned DNA. Transcription rates were quantifiated from the slot blots by densitometric scanning and densities in arbitrary units shown as bar graphs after correction for the appropriate vector background controls A, untrested rate; B, AIA treated; C, 5-aminolevulinate treated; D, AIA and 5-aminolevulinate treated. DNA clones: lane 1, 5-aminolevulinate synthase; lane 2, Milimpis, lane 3, chicken factin; lane 4, chicken serum albumin; lane 5, pBR322. lane 5, pBR322

Our results show that administration of AIA alone for 12 h resulted in a 10-fold increase in the transcriptional rate of the 5-aminolevulinate synthase gene (see Fig. 9). This correlated with a 7-fold increase in hepatic 5-aminolevulinate synthase mRNA levels measured at this time (see Fig. 8; panel A).

The effect of 5-aminolevulinate on the transcription rate

of the 5-aminolevulinate synthase gene was investigated. Administration of this compound to untreated rate significantly reduced the low basal transcriptional rate of the 5-aminolev ulinate synthese gene (Fig. 9). Administration of 5-aminolev ulinate to AIA-treated rats prevented the drug-induced in crease in the rate of gene transcription (Fig. 9). A corresponding reduction in the level of hepatic 5-aminolevulinate synthese mRNA was observed (Fig. 8, panel A). Throughout this work, the transcriptional rate of the serum albumin gene measured as a control was unchanged although that of the β actin gene was slightly elevated by either AIA or 5-aminolevulinate treatment (Fig. 9). The reason for the latter is unknown. Other control experiments established that the presence of 5-aminolevulinate (50 μ M) or hemin (0.01–10 μ M) in the transcription reaction had no effect on any of the genes under test. Overall, these results demonstrate that in the liver, AIA and 5-aminolevulinate treatment modulates 5-aminolevulinate synthese mRNA levels by altering the rate of gene transcription.

DISCUSSION

The early work of Granick (3) established that in chick embryos drug inducibility of hepatic 5-aminolevulinate synthase activity is prevented by the simultaneous administration of heme. The mechanism of this has remained a central

question ever since. It was originally postulated that home and drugs compete for a site on a gene-controlling protein. This was rendered improbable by the work of Srivastava et al (23) who showed that heme repression appeared to be the sole control and that drug induction was probably a secondary consequence of heme removal (reviewed in Ref. 1). Since the original postulate, it has been variously claimed that heme works at the translational and transcriptional level, and considerable confusion has existed (1). This was, in part, due to studies being done at the enzymic level and to the complication that hemin prevents entry of newly synthesized 5-aminolevulinate synthase into the mitochondrion (1).

In this work we have studied 5-aminolevulinate synthese control at the mRNA level. To this end a cDNA clone for rat liver 5-aminolevulinate synthase has been isolated and sequenced. Using probes derived from this, control of 5-aminolevulinate synthese mRNA in various rat tissues has been

examined

The first question investigated was whether there exists in rat a single mRNA for 5-aminolevulinate synthase or a multiplicity of them. This question arises from the suggestion that a family of 5-aminolevulinate synthase genes exists in chicken (6). The results here give compelling evidence that the 5-aminolevulinate synthase mRNA in all rat tissues examined is the same and that only a single species exists. This is in keeping with our recent conclusion that the erythroid 5aminolevillinate synthase in chicken is coded for by the same gene as that in the liver (11). The work also shows that the rat liver 5-aminolevulinate synthese mRNA present during fetal development is indistinguishable on Northern blots from

Although drug induction of 5-aminolevulinate synthese in liver is well known, the inducibility in other tissues has not been well documented. We show here that 5-aminolevulinate synthase mRNA is induced by AIA only in the liver and kidney of rat. Correlating with this, the level of the pheno-barbital inducible cytochrome P 450 b/e mRNAs are elevated by AIA specifically in these tissues. Interestingly, 5-eminolevulinate synthase mRNA in testis is induced by HCG which also induces tissue specific cytochrome P-450 proteins (24). These results support the proposal (1) that inducibility of 5-aminolevulinate synthase is a secondary consequence of heme depletion due to induction of cytochrome P-450 apoprotein which takes up heme as a prosthetic group.

A basal level of 5-aminolevulinate synthase mRNA was detected in all rat tissues examined. A question which has not been addressed previously is whether hame controls the basal level of 5-aminolevulinate synthase mRNA or only the drugstimulated increase and also whether this hame control is confined to liver. The results in this paper establish that heme repression of 5-aminolevulinate synthase mRNA levels occurs in all rat tissues studied, with the exception of erythroid spleen, with both besal and induced levels being affected. In erythroid spleen the level of 5-aminolevulinate synthase mRNA was elevated. Possibly this is indirectly due to the induction of hame oxygenase (25). A reservation in this work is that possibly for cell permeability reasons, administered hemin affects only liver 5-aminolevulinate synthese mRNA levels. However, administered 5-aminolevulinate (or its methyl ester in the case of brain) lowers the 5-aminolevulinate synthase mRNA level in all tissues except spleen. It seems most probable that repression by heme is being observed since there is no evidence that 5-aminolevulinate itself has a regulatory role, and it is known that injected 5-aminolevulinate is rapidly converted to heme in many tissues (22).

G. Srivastava, unpublished data

A final question concerns the level at which heme control is exerted. We have conclusively established that in liver, heme regulates 5-aminolevulinate synthese mRNA levels by acting predominantly, if not exclusively, to inhibit transcription of the 5-aminolevulinate synthase gene.

This work places the 5-aminolevulinate synthase gene in a small group of animal genes (26, 27) known to be negatively controlled by a metabolic end product. The molecular basis for the regulation of the 5-aminolevulinate synthese gene is an important problem to be investigated.

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